Transformation of Cowpea Vigna unguiculata Cells with an Antibiotic Resistance Gene Using a Ti-Plasmid-Derived Vector

Hille, Jacques; Goldbach, Rob

Published in:
Plant Science

DOI:
10.1016/0168-9452(86)90166-4

Publication date:
1986

Plant Science, 44 (1986) 37—46
Elsevier Scientific Publishers Ireland Ltd.

TRANSFORMATION OF COWPEA *VIGNA UNGUICULATA* CELLS WITH AN ANTIBIOTIC RESISTANCE GENE USING A Ti-PLASMID-DERIVED VECTOR

JUAN A. GARCIA*, JACQUES HILLE and ROB GOLDBACH
Department of Molecular Biology, Agricultural University, De Dreijen 11, 6703 BC Wageningen (The Netherlands)

(Received December 2nd, 1985)
(Revision received January 21st, 1986)
(Accepted January 21st, 1986)

A chimaeric antibiotic resistance gene was transferred to cowpea *(Vigna unguiculata)*, a member of the legume family. This transfer was established by inoculating cowpea leaf discs with an *Agrobacterium tumefaciens* strain harboring a Ti-plasmid-derived vector that contained two copies of a chimaeric kanamycin resistance gene. By culturing the leaf discs in selective medium kanamycin resistant callus tissue was obtained. Transformation of this callus tissue was confirmed by the detection of nopaline synthase activity and by Southern blot hybridization revealing the integration of the kanamycin resistance gene in the plant DNA.

Key words: Vigna unguiculata; leaf disc transformation; *Agrobacterium tumefaciens;* kanamycin resistance; Southern blot hybridisation

Introduction

After the development of plant gene vectors derived from *A. tumefaciens* Ti-plasmids, successful transfer of cloned genes has been reported for a number of plant species, including tobacco, petunia and tomato [1—3]. In these cases a chimaeric antibiotic resistance gene, provided with suitable regulation signals active in plants, has been chosen as the gene to be transferred, since this allows convenient selection of transformed tissues.

Until now transfer of cloned genes to members of the large and economically important *Leguminosae* family has not been reported. One of the reasons for the lag in engineering leguminous plants has been the difficulty in regenerating entire plants from protoplasts or callus tissue. Despite many attempts, so far successful regeneration has been reported for only a few members of the legume family, including *Medicago* sps. [4,5] and *Trifolium repens* [6], while a single report describes the regeneration of moth bean (*V. aconitifolia)* from mesophyll protoplasts [7].

Since we are interested in the interactions between cowpea mosaic virus (CPMV) [8,9] and cowpea, *V. unguiculata*, its natural host, we have investigated whether foreign genes can be stably introduced and expressed in this plant species. This would open the way to study host-virus interactions by introducing parts of the CPMV genome in the host chromosomal DNA.

For cowpea, no convenient regeneration procedure is currently available. We have therefore tested the possibility of studying the expression of introduced genes on callus tissue level, thereby by-passing the general

*Present address: Centro de Biologia Molecular (CSIC-UAM) Universidad Autonoma, Cantoblanco 28049, Madrid, Spain.

Abbreviations: APT, amino glycoside phosphotransferase; bp, base pairs; CPMV, cowpea mosaic virus; CTAB, cetyltrimethylammonium bromide; kb, kilobasepair; SDS, sodium dodecyl sulphate; SSC, 0.15 M sodium chloride/0.015 M sodium citrate.

0168-9452/86/$03.50

difficulty of whole plant regeneration with legumes.

In this paper, we report the successful and stable introduction of a chimaeric antibiotic resistance gene in cowpea cells by adopting the recently developed leaf disc transformation technique [3,10], and the subsequent production of transformed callus tissue in which the expression of this gene can be readily studied.

Materials and methods

Bacterial strains and plasmids

The *A. tumefaciens* strains LBA1010, containing the octopine type plasmid pTiB6 [11] and LBA958, containing the nopaline type plasmid pTiC58 [12] were used for infection of cowpea plants. Strain C58C1 harboring the non-oncogenic Ti-plasmid pGV3850::1103neo(dim) which contains two identical chimaeric kanamycin resistance genes was used for the leaf disc transformation experiment. Plasmid pGV3850::1103neo(dim) was a gift of Dr. M. van Montagu and Dr. L. Herrera-Estrella and has been described previously [22].

Tumour induction assay on cowpea

Seven-day-old cowpea plants were inoculated with *A. tumefaciens* by dipping a toothpick in a colony of the *A. tumefaciens* strain to be tested and puncturing the stem. Plants were maintained in a growth chamber at a relative humidity of 80—90% and a daily cycle of 14 h light (and 28°C) and 10 h dark (and 22°C).

Transformation of cowpea

For transformation of cowpea cells a modification of the leaf disc transformation procedure described by Horsch et al. [3] was used. Discs punched from primary leaves of sterile grown cowpea plants (6 days old) were submerged for 30 s in a 1:100 dilution (in GS3 medium, see below) of an overnight culture of *A. tumefaciens* containing pGV3850::1103neo(dim), washed briefly, and blotted dry on filter paper. After inoculation the discs were incubated at 25°C on nurse culture plates containing the callus-inducing medium GS3 [7] solidified with 0.8% agar, a feeder layer of *Petunia* 'commanche-albino' cells and a sheet of Whatman 5 filter paper. After 2 days the discs were transferred to petri dishes with the same medium containing the antibiotics cefotaxime (200 μg/ml) and vancomycin (200 μg/ml) to stop bacterial growth, and antibiotic G418 (50 μg/ml) for selection of transformed plant cells. After 2—4 weeks callus tissue was obtained, mainly from the edges of the leaf discs, and subcultured every 20 days on plates containing kanamycin (100 μg/ml) instead of antibiotic G418. After 6 transfers cefotaxime and vancomycine were omitted from the medium and callus tissue culturing was maintained at 25°C with subculturing each 2 or 3 weeks.

DNA preparation and Southern blot analysis

Total DNA from *A. tumefaciens* containing plasmid pGV3850::1103neo(dim) was isolated according to Ooms et al. [13] and plant DNA following the CTAB procedure of Murray and Thompson [14]. For Southern blot analysis [15] 5 μg of total plant DNA was digested with approx. 100 units of each of the restriction endonucleases employed. The DNA was applied to a 1% agarose gel containing TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA). Specific probes were prepared from M13 templates using an M13 sequencing primer and the Klenow fragment of DNA polymerase I [16]. Unreacted nucleotides were removed by chromatography on a Sephadex G50 column. The specific activity of the probes was approx. 10^8 cpm/μg. Probe A consisted of the 352 base pairs (bp) PstI-SphI fragment from the aminoglycoside phosphotransferase (APT)-II gene of Tn5 [17] inserted in M13mp19 [18] and probe B consisted of a 1.4 kb EcoRI-SalI fragment containing the nopaline synthase promoter, the APT-II gene of Tn5 and the nopaline synthase

terminator inserted in M13mp8 [19]. Prehybridization was performed for 16 h at 65° in 5× Denhardt solution [20], 40 mM Tris—HCl (pH 6.8), 5× 0.15 M sodium chloride/0.015 M sodium citrate (SSC), 1 mM EDTA and 150 μg/ml of denatured salmon sperm DNA. Hybridization was carried out for 48 h at 65°C in the prehybridization solution containing about 5 ng/ml of denatured, ^{32}P-labeled DNA probe, prepared as described above. Filters were washed twice for 30 min in subsequently 5× SSC, 1 mM EDTA, in 2× SSC, 0.1% sodium dodecyl sulphate (SDS) and in 0.1× SSC, 0.1% SDS at 65°C and, finally, autoradiographed at —70°C using an intensifier screen.

Growth rate of cowpea calli on medium containing increasing amounts of kanamycine

Callus samples (6 individual calli per sample) were preincubated for 3 weeks in petri dishes containing GS3 medium with 0.8% agar and a given concentration of kanamycin. After this period the samples were weighed and transferred to new plates containing the same medium and kanamycin concentration. Incubation was continued for 16 days at 25°C with a daily cycle of 16 h light and 8 h dark. After this period tissue was collected, again weighed and the growth rate determined. Each estimate was the average of at least two plates each containing 6 calli.

Results

Infection of cowpea with Agrobacterium tumefaciens

Since no data were available indicating that cowpea is susceptible to *A. tumefaciens*, stems of 7-day-old cowpea plants were inoculated with *A. tumefaciens* strain LBA1010 harboring a Ti-plasmid of the octopine type (pTiB6) or strain LBA958 harboring a Ti-plasmid of the nopaline type (pTiC58). In both cases infection induced the formation of crown gall tumours at the wound site as shown for LBA1010 (pTiB6) in Fig. 1. The production of opines in these tumours (data not shown) demonstrated that transformation had occurred and that in principle foreign genes can be transferred to cowpea using Ti-plasmid-derived vectors. Moreover, these results indicated that for the transformation of cowpea chimaeric antibiotic resistance genes can be used that are supplied with regulatory sequences from either the octopine or nopaline synthase gene.

Transformation of cowpea cells using Ti-plasmid pGV3850::1103neo(dim)

Since normal cowpea callus is sensitive to kanamycin, we used a chimaeric gene in the transformation studies that confers kanamycin resistance on transformed plant cells.

To transfer and integrate such a resistance gene into the cowpea chromosomal DNA the Ti-plasmid-derived vector pGV3850::1103neo(dim) (Fig. 2) was employed. In this vector all the oncogenic functions have been deleted and replaced by pBR322 sequences and two copies of a chimaeric kanamycin resistance gene, consisting of the nopaline synthase promoter, the coding region of the aminoglycoside phosphotransferase (APT-II) gene of Tn5 and the poly(A) signal region of the octopine synthase gene.

To transform cowpea cells we adopted the leaf disc transformation method developed by Horsch et al. [3] and modified as described in Materials and methods. In initial experiments transformant cells were selected by culturing in 2Z regeneration medium that induces shoot formation with tobacco and tomato (Koornneef et al., Plant Mol. Biol., submitted), containing antibiotic G418 (50 μg/ml). Since attempts to obtain regeneration to whole cowpea plants failed, in later experiments GS3 medium [7], also containing antibiotic G418, was used to optimize the growth of callus tissue. Following this approach culturing at 25°C yielded cowpea calli able to grow in the presence of kanamycin (50 μg/ml) or antibiotic G418 (50 μg/ml),

Fig. 1. Tumor formation on a cowpea (*V. unguiculata*) stem 3 weeks after inoculation with *A. tumefaciens* LBA1010 (pTiB6).

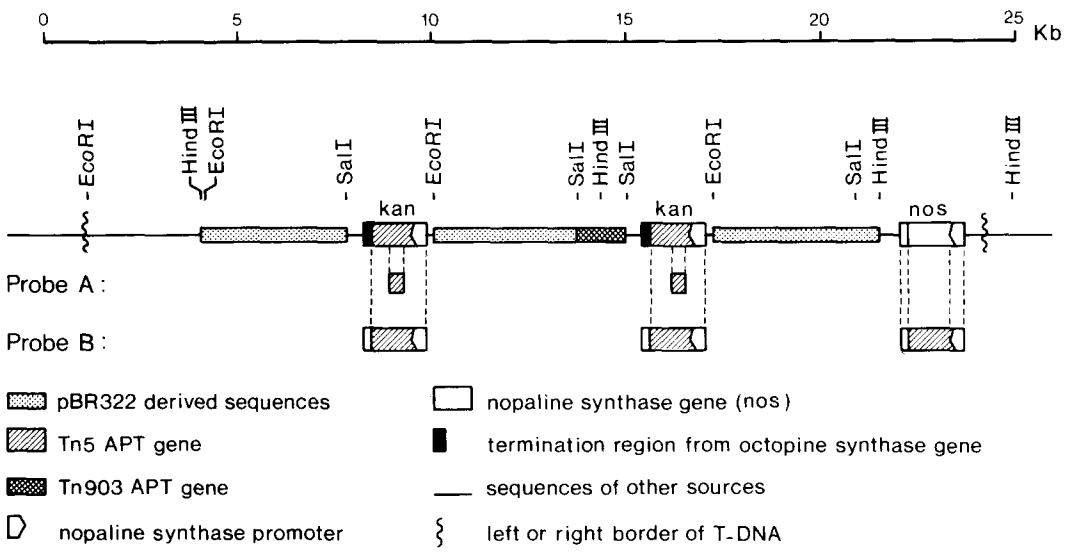

Fig. 2. Physical map of the T-region of plasmid pGV3850::1103neo(dim). Restriction sites and gene alignment have been taken from Czernilofsky et al. [22]. The probes used in the hybridization experiments are drawn below, dashed lines connecting the homologous regions (for details see Materials and methods).

while control calli (non-transformed), also generated from leaf discs, were not able to grow in the presence of either antibiotic (Fig. 3). To verify whether these antibiotic-resistant calli were faithfully transformed they were assayed for their nopaline content. The resistant cowpea calli tested indeed contained nopaline (Fig. 4), hence these calli originated from transformed mesophyll cells.

Genomic analysis of the kanamycin-resistant calli

To verify the chromosomal integration of the kanamycin resistance gene genomic DNA of small pools of resistant calli was isolated and digested with restriction enzymes SalI and EcoRI. The separated restriction fragments were blotted onto nitrocellulose filters and hybridized to ^{32}P-labeled probes, specific to different parts of the chimaeric kanamycin gene as indicated in Fig. 2. In Fig. 5 panel A the genomic fragments were hybridized to a 0.35 kilobasepairs [kb] fragment specific to the ATP-II gene of Tn5 (denoted probe A in Fig. 2). With DNA from callus tissue, cultivated for 20 weeks in GS3 medium containing kanamycin a hybridization pattern was obtained, with a single predominant band at a position of approx. 2.3 kb (Fig. 5A, lane 2), while DNA from non-transformed calli did not show any hybridization signal (Fig. 5A, lane 5). This hybridizing fragment is identical to the APT-gene-containing fragment generated with EcoRI and SalI from the bacterial plasmid pGV3850::1103neo(dim) (cf. Fig. 2 and the hybridization in Fig. 5A, lane 1). Similarly, when a 1.4 kb ^{32}P-probe was used containing sequences of both the APT (Tn5) gene and the nopaline synthase promoter (probe B, see Fig. 2) DNA digests from transformed calli again gave a predominant, hybridizing band at a position of ~ 2.3 kb, comigrating with a hybridizing fragment in the bacterial DNA digest (cf. lanes 1 and 2 in Fig. 5B). The extra 5 kb band in the bacterial DNA digest is a border fragment containing the right-hand border sequence and the nopaline synthase gene (Fig. 2). The absence of this fragment in the digest of cowpea DNA should be caused

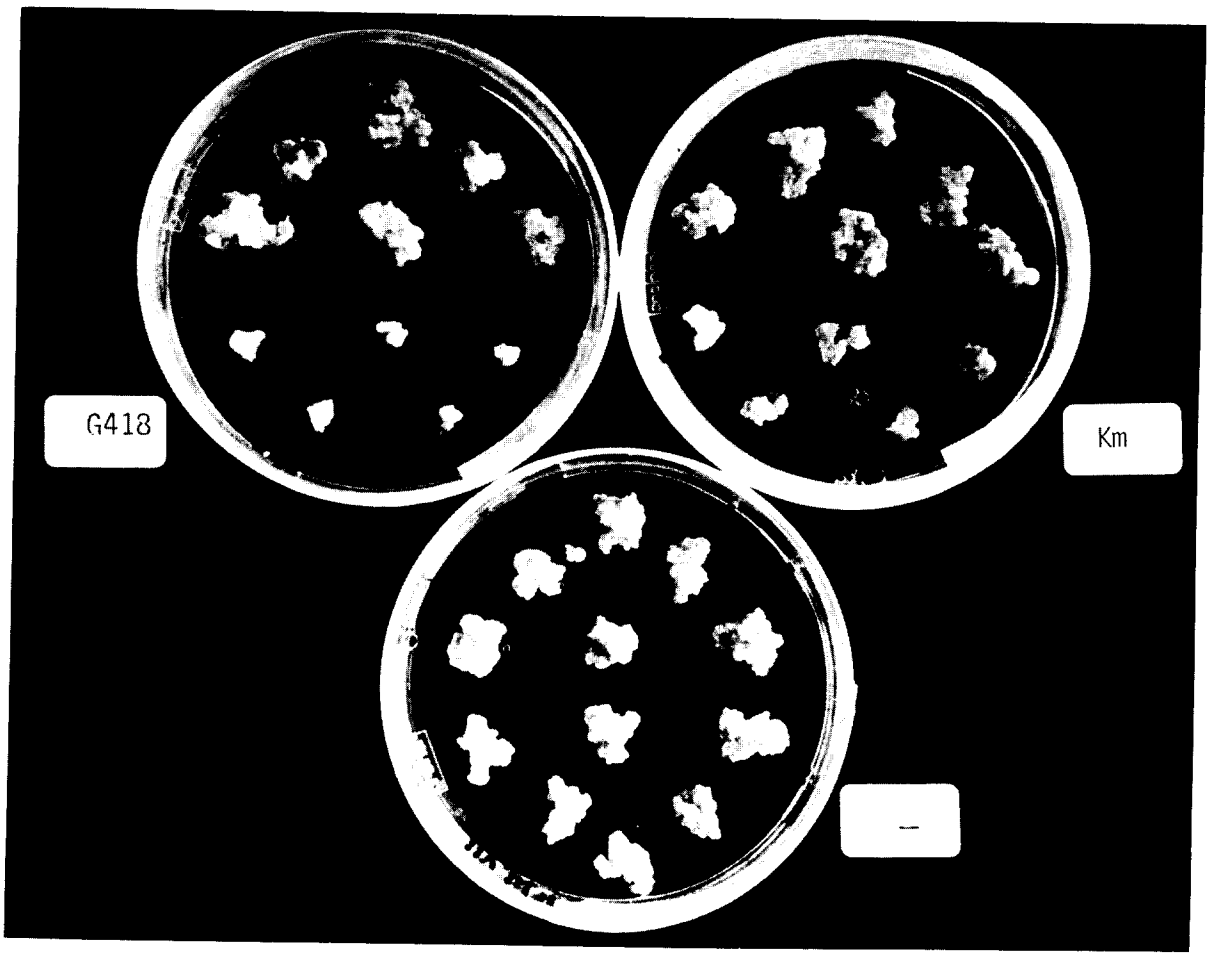

Fig. 3. Cowpea calli transformed with *A. tumefaciens* carrying plasmid pGV3850::1103neo(dim) (upper half of the petri dishes) or non-transformed (lower half of the petri dishes) grown in GS3 medium containing 50 μg/ml kanamycin (K_m), 50 μg/ml G418 (G418) or without antibiotic (—).

by chromosomal integration of the T-DNA part of pGV3850::1103(neo)dim), by which the nopaline synthase gene, located near the right border sequence (Fig. 2), appears on different junction fragments in the genomic DNA digest, depending on the sites of integration.

The integration of the kanamycin resistance gene in cowpea DNA was verified in a third hybridization experiment in which the genomic DNA of transformed calli was digested with EcoRI and HindIII and hybridized to probe B. While the pattern of pGV3850::1103neo(dim) plasmid, also digested with EcoRI and HindIII, contained three DNA bands hybridizing to this probe (at positions 6.0 kb, 3.4 kb and 2.9 kb) the digest of DNA from transformed calli was clearly different, showing only two of these bands (at positions 6.0 and 2.9 kb), together with a series of less predominant bands (cf. lanes 1 and 2 in Fig. 5C). The difference between the hybridization patterns of bacterial and callus DNA definitely demonstrates that the T-DNA region of pGV3850::1103neo(dim) indeed had been integrated

Fig. 4. Electropherogram of the products formed by nopaline synthase activity in extracts of cowpea calli transformed by *A. tumefaciens* harboring plasmid pGV3850::1103neo(dim) or non-transformed (control). The assay was performed as described by Otten and Schilperoort [23]; reaction mixtures were spotted at times 0 and 16 h.

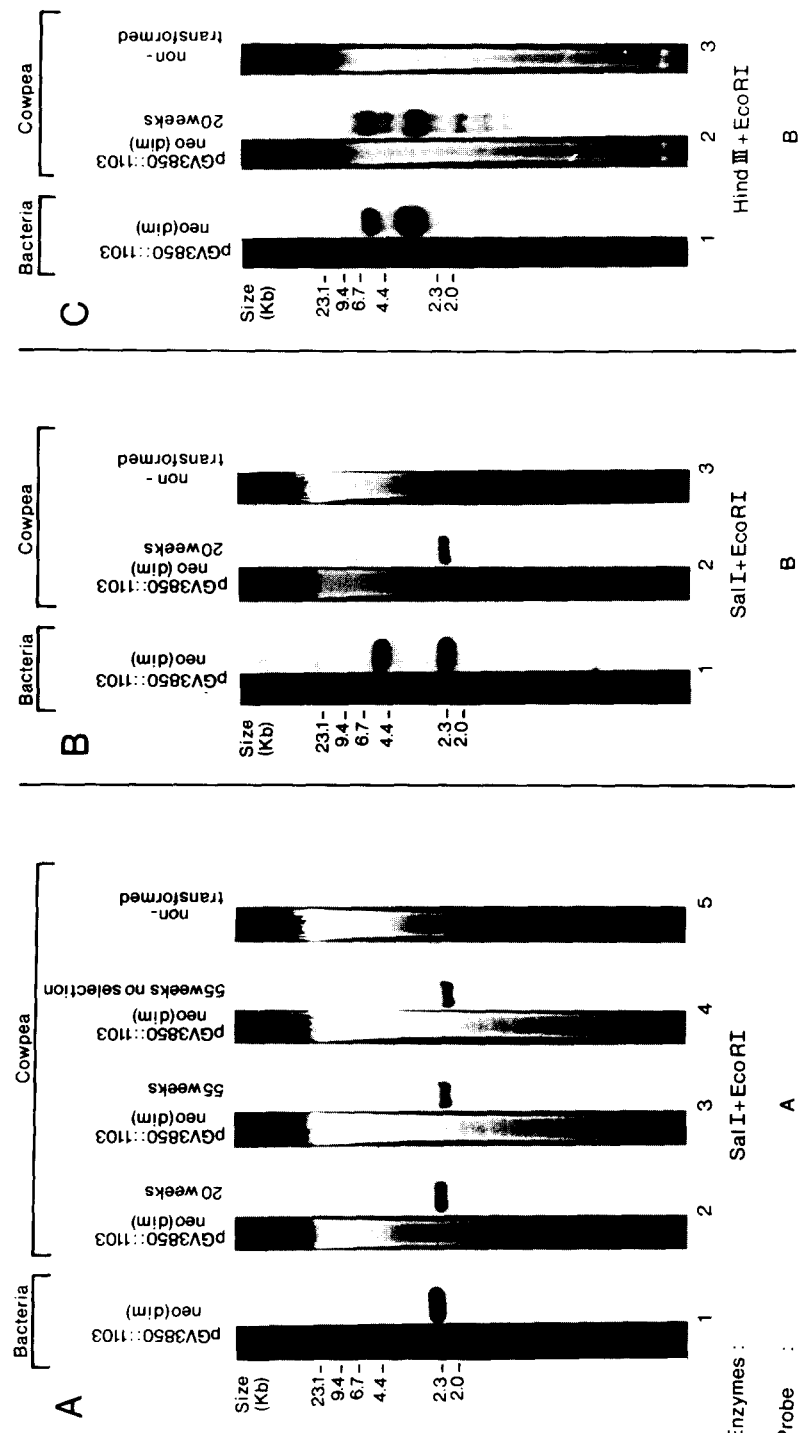

Fig. 5. Southern blot analysis of DNA from cowpea calli transformed by *A. tumefaciens* carrying pGV3850 ::1103neo(dim). DNA isolated from cowpea calli (both transformed and non-transformed) and DNA from bacteria containing the plasmid were double-digested with SalI/EcoRI (panels A and B) or HindIII/EcoRI (panel C). The fragments were separated in 1% agarose gels, blotted onto nitrocellulose filters and subsequently hybridized to radioactive probe A (panel A) or probe B (panels B and C). Each lane contains at the left the ethidium bromide stained gel and at the right the autoradiograph of the nitrocellulose blot after hybridization. The origin of the DNA and the culturing time of the calli are indicated at the top of each lane. No selection (panel A, lane 4) means that calli were grown for 40 weeks in the absence of kanamycin prior to analysis. The position and size of fragments generated by HindIII digestion of λDNA are shown at the left side of each panel.

into the DNA of cowpea. In the patterns of Fig. 5C the hybridizing fragments of 6.0 kb and 2.9 kb correspond to the two chimaeric kanamycin resistance genes while the fragment of 3.4 kb, missing in the genomic digest, corresponds to the nopaline synthase gene on the right-hand border fragment of pGV3850::1103neo(dim) (cf. Fig. 2). The replacement of the 3.4 kb band by a number of different minor bands indicates that integration occurred at various sites in the DNA of the cowpea calli tested.

To test the stability of the integration, cowpea calli, transformed with the T-region of pGV3850::1103neo(dim), were grown for 55 weeks either in the presence or absence of kanamycin. After this period the genomic DNA was isolated from both callus lines and analyzed by Southern hybridization for the presence of the kanamycin resistance genes. The identical hybridization signal obtained in both cases (cf. Fig. 5A, lanes 3 and 4) indicates that the integration of the kanamycin genes is stable even after a long period of non-selective growth.

Phenotype of transformed cowpea callus tissue

Having established that the chimaeric kanamycin resistance genes have been integrated we next investigated the level of kanamycin resistance of transformed cowpea calli. The growth rate of kanamycin-resistant and control (non-transformed) calli was determined in GS3 medium containing increasing amounts of kanamycin. While non-transformed calli showed a very drastic decrease in growth in the presence of kanamycin (no growth on medium containing 100 $\mu g\ ml^{-1}$ kanamycin or more) the transformed calli showed no decrease in growth rate until 200 $\mu g\ ml^{-1}$ kanamycin and only a slow decrease in growth rate at higher concentrations (Fig. 6). Even at 500 μg/ml kanamycin the transformed calli were able to grow at a rate more than 50% of the rate measured in the absence of kanamycin (Fig. 6). From these results we conclude that the transferred chimaeric kanamycin genes are efficiently expressed in the transformed cowpea calli.

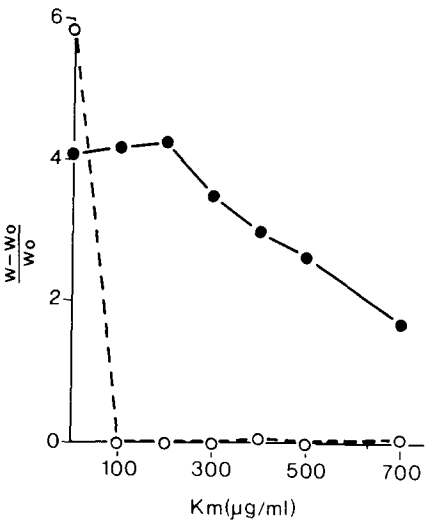

Fig. 6. Growth of cowpea calli on kanamycin-containing medium. •——•, cowpea calli transformed by *A. tumefaciens* containing pGV3850::1103neo(dim); ○——○, non-transformed cowpea calli; Wo, initial weight (~250 mg); W, final weight after 16 days.

Discussion

In this paper we demonstrate the possibility of transforming cowpea (*V. unguiculata)* cells using an *A. tumefaciens* strain harboring a non-oncogenic, Ti-plasmid-derived vector and applying the recently developed [3,10] leaf disc transformation technique. To our knowledge this is the first report on successful transformation of a member of the economically important *Leguminosae* family. Although we have not been able to regenerate whole plants from the transformed callus tissue obtained — a problem which seems to be typical for most leguminous plants — the procedure described here efficiently yields transgenic non-tumorous callus tissue, that lends itself very well to molecular analyses. Thus a chimaeric kanamycin resistance gene from plasmid pGV3850::1103neo-(dim), previously shown to be expressed

in tobacco cells [21,22], has been transferred into cowpea cells rendering callus tissue resistant to kanamycin. The transformed calli obtained have maintained their antibiotic resistant phenotype for more than 1 year now. Even when these calli are cultured for 40 weeks in non-selective medium (no antibiotic added) they exhibit virtually undiminished resistance to kanamycin and G418. Southern blot analysis of the DNA from such tissue confirms that integrated kanamycin resistance genes have been conserved during that period. This stable phenotype indicates that the kanamycin resistance gene can be exploited as marker in the selection of transformed cowpea cells.

Experiments are currently in progress to integrate full-length DNA copies of the two genomic RNAs of CPMV into the genomic DNA of cowpea, with the purpose to study host-virus interactions. This kind of experiment has only become feasible by establishing that the kanamycin resistance gene can be used as marker for selecting transformed cowpea cells.

Acknowledgement

The authors wish to thank Dr. M. van Montagu and Dr. L. Herrera-Estrella for the kind gift of plasmid pGV3850::1103neo-(dim) and for sending a manuscript prior to publication, Niels Galjart for the construction of M13 probe B, Rijndert de Fluiter for artwork and Gré Heitkönig for skilful typing of the manuscript, J.A.G. was supported by an EMBO long-term fellowship.

References

1 R.B. Horsch, R.T. Fraley, S.G. Rogers, P.R. Sanders, A. Lloyd and N. Hoffmann, Science, 223 (1984) 496.
2 M. de Block, L. Herrera-Estrella, M. van Montagu, J. Schell and P. Zambryski, EMBO J., 3 (1984) 1681.
3 R.B. Horsch, J.E. Fry, N.L. Hoffmann, D. Eichholz, S.G. Rogers and R.T. Fraley, Science, 227 (1985) 1229.
4 K.N. Kao and M.R. Michayluk, Z. Pflanzenphysiol., 96 (1980) 135.
5 L.B. Johnson, D.L. Stuteville, R.K. Higgins and D.Z. Skimmer, Plant Sci. Lett., 20 (1981) 297.
6 S.S. Bhojwani and D.W.R. White, Plant Sci. Lett., 26 (1982) 265.
7 N.S. Shekhawat and A.W. Galston, Plant Sci. Lett., 32 (1983) 43.
8 R. Goldbach, H. Franssen, G. Rezelman, A. van Kammen, J. Verver and P. van Wezenbeek, Structure and genetic organization of cowpea mosaic virus RNAs, in: H.D. Robertson, S.H. Howell, M. Zaitlin and R.L. Malmberg (Eds.), Plant Infectious Agents, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1983, p. 100.
9 R. Goldbach and A. van Kammen, Structure, replication and expression of the bipartite genome of cowpea mosaic virus, in: J.W. Davies (Ed.), Molecular Plant Virology, Vol. I, CRC Press, Boca Raton, 1985, p. 83.
10 D. Tepfer, Cell, 37 (1984) 959.
11 B.P. Koekman, P.J.J. Hooykaas and R.A. Schilperoort, Plasmid, 7 (1982) 119.
12 P.J.J. Hooykaas, H. den Dulk-Ras, G. Ooms and R.A. Schilperoort, J. Bacteriol., 143 (1980) 1295.
13 G. Ooms, P.J.J. Hooykaas, G. Moolenaar and R.A. Schilperoort, Gene, 14 (1981) 33.
14 M.G. Murray and W.F. Thompson, Nucl. Acids Res., 19 (1980) 4321.
15 E.M. Southern, J. Mol. Biol., 98 (1975) 503.
16 J. Messing, R. Crea and P.H. Seeburg, Nucl. Acids Res., 9 (1981) 309.
17 E. Beck, G. Ludwig, E.A. Auerswald, B. Reiss and H. Schaller, Gene, 19 (1982) 327.
18 J. Norrander, T. Kempe and J. Messing, Gene, 26 (1983) 101.
19 J. Messing and J. Vieira, Gene, 19 (1982) 269.
20 T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular cloning, A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1982.
21 K. Hain, P. Stabel, A.P. Czernilofsky, H.H. Steinbiss, L. Herrera-Estrella and J. Schell, Mol. Gen. Genet., 199 (1985) 161.
22 A.P. Czernilofsky, R. Hain, L. Herrera-Estrella, H. Lörz, B.J. Baker and J. Schell, DNA, (1986) in press.
23 L.A.B.M. Otten and R.A. Schilperoort, Biochim. Biophys. Acta, 527 (1978) 497.